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NOTES

Detection of Mycotoxins by Thin-Layer Chromatography: Application to Screening of Fungal Extracts

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A convenient thin-layer chromatographic screening procedure for the detection of 18 mycotoxins is described.

Examination in our laboratory of fungi isolated from foods and feeds for their ability to produce aflatoxins (15) has been expanded to include other mycotoxins. This has been made possible by thin-layer chromatography (TLC) with suitable general solvent systems and only one initial spray reagent. We can thus detect the following toxins: aflatoxins B_1, B_2, G_1, and G_2 (3); ochratoxin A (14); aspertoxin (8); luteoskyrin (13); zearalenone [F-2 (6)]; 4-acetamido-4-hydroxy-2-butenoic acid γ-lactone (18); diacetoxyscirpenol (1) and its 8-(3-methylbutyryloxy) derivative [T-2 toxin (5)]; and nivalenol and its 4-*O*-acetate (11), in addition to several antibiotics now regarded as mycotoxins, namely gliotoxin, citrinin, patulin, penicillic acid, and sterigmatocystin (4). These mycotoxins are produced mainly by species of *Aspergillus*, *Penicillium*, or *Fusarium* but are not necessarily restricted to any one species or genus.

TLC was carried out in subdued light. Thin layers (0.3 mm) of Adsorbosil 5 silica gel (Applied Science Laboratories, Inc., State College, Pa.) were activated at 110 C for 2 hr. Five-microliter amounts of standard solutions of each toxin in an appropriate organic solvent were spotted and developed for a distance of 15 cm in the following solvent systems, with normal saturation: toluene–ethyl acetate–90% formic acid (6:3:1; TEF) and benzene–methanol–acetic acid (24:2:1; BMA). Toxins were visualized in visible or ultraviolet light (Chromato-Vue cabinet, Ultra-Violet Products, Inc., San Gabriel, Calif.), before and after spraying the plate with a freshly prepared mixture of 0.5 ml of *p*-anisaldehyde in 85 ml of methanol containing 10 ml of glacial acetic acid and 5 ml of concentrated sulfuric acid (16) and then heating at 130 C for 8 to 20 min. The shorter heating time was better for fluorescence development. Typical R_F values and colors observed are shown in Table 1.

The advantage of the acidic solvent systems is that all of the toxins migrate, although nivalenol stays close to the origin and citrinin and luteoskyrin streak. Fluorescence colors of certain mycotoxins in ultraviolet light are well known (10). The anisaldehyde spray allows detection of the nonfluorescent toxins. After the spray treatment, the blue fluorescence of T-2 toxin and penicillic acid in longwave ultraviolet light permits detection of less than 0.2 and 0.01 μg, respectively, on the TLC plate. These limits are much lower than those previously reported (9, 17). Detection limits for nivalenol, nivalenol 4-*O*-acetate, diacetoxyscirpenol, 4-acetamido-4-hydroxy-2-butenoic acid γ-lactone, and gliotoxin are of the order of 0.2 μg. Replacement of methanol in the spray reagent by ethanol improves the detection of patulin, which then forms a reddish spot (detection limit, 0.1 μg).

Once the order of migration and detection colors are familiar, standard toxins that separate in each of the solvent systems can be mixed and spotted together. This is commonly done with the four aflatoxins.

With the foregoing detection procedure, we evaluated our semimicro culture technique (15)

TABLE 1. *Thin-layer chromatography of mycotoxins*

Mycotoxin	Concn[a] (μg/ml)	R_F		Color			Color after spray treatment		Alternative spray reagent
		Solvent system		Visible light	Ultraviolet light		Visible light	Longwave ultraviolet light	
		TEF	BMA		Longwave	Shortwave			
Citrinin	10	0.16–0.48[b]	0–0.20[b]		Yellow[c]	Yellow		Yellow-green	
Luteoskyrin	1,000	0–0.47[b]	0–0.23[b]	Yellow[c]	Yellow		Orange	Yellow[c]	
Nivalenol	1,000	0–0.02[b]	0–0.01[b]				Gray	Light-brown	
4-Acetamido-4-hydroxy-2-butenoic acid γ-lactone	1,000	0.10	0.03				Gray[d]		
Kojic acid	1,000	0.16	0.03						$FeCl_3$
Aflatoxin G_2	0.2	0.17	0.13		Green[c]	Faint green		Blue	
Nivalenol acetate	1,000	0.19	0.09				Brown[c]	Brown	
Aflatoxin G_1	1	0.23	0.14		Green[c]	Faint green		Blue	
Aflatoxin B_2	0.2	0.26	0.20		Blue[c]	Faint blue		Pink	
Aflatoxin B_1	1	0.31	0.23		Blue[c]	Faint blue		Pink	
Diacetoxyscirpenol	1,000	0.33	0.24				Pink[c]	+[e]	
Aspertoxin	50	0.35	0.13		Bright yellow	Bright yellow		Green-yellow	
T-2 toxin	100	0.36	0.28				Gray-pink	Blue[c]	
Patulin	200	0.41	0.21				Faint brown	Yellow[c]	Phenylhydrazine (9)[c]
Penicillic acid	200	0.47	0.22				Green	Blue[d]	
Gliotoxin	1,000	0.53	0.39				Yellow-brown[e]	Light blue	Silver nitrate (12)
Ochratoxin A	10	0.55	0.35		Green	Green[c]		Faint blue	
Zearalenone	50	0.78	0.42		Faint blue	Blue-green[c]	Faint brown	Faint yellow	
Sterigmatocystin	50	0.85	0.75		Red-brown			Yellow[d]	

[a] In chloroform solution, except for citrinin and aflatoxins (in benzene-acetonitrile, 98:2), nivalenol (in ethyl alcohol), and kojic acid (in ethyl acetate).
[b] Streak.
[c] Preferred means of detection.
[d] Improved sensitivity if TLC plate still hot.
[e] Color variable.

TABLE 2. *Production of mycotoxins by representative fungi*

Fungus	Strain number	Mycotoxins	Media
Penicillium patulum	ATCC 18172	Patulin	YES
P. urticae	NRRL 1953	Patulin	YES
P. terlikowski	HLX 136[a]	Gliotoxin	CZ + YE
P. viridicatum	69-22[b]	Citrinin, ochratoxin A	YES
Aspergillus flavus	MRE 2	Aspertoxin; aflatoxins B_1, B_2, G_1, G_2	YES, MYE
A. flavus (4 strains)		Kojic acid; aflatoxins B_1, B_2	YES
A. sulphureus	ATCC 18413	Penicillic acid; aflatoxins B_1, G_1; ochratoxin A	YES
A. versicolor	CMI 49124	Sterigmatocystin	CZ + YE, MYE
A. versicolor	CMI 16139	Sterigmatocystin	YES, MYE
A. versicolor	64-45[c]	Sterigmatocystin	YES, MYE, CZ + YE
Cochliobolus nodulosus (*Bipolaris* state)		Sterigmatocystin	YES
Fusarium sp.	Mapleton 10[d]	Zearalenone	Moist rice[e]
Fusarium sp.	69-15[b]	T-2 toxin	YES[e]
Fusarium sp.	69-17[b]	T-2 toxin	YES[e]
Fusarium sp.	66-57-3[b]	Diacetoxyscirpenol	YES

[a] National Research Council of Canada, Halifax.
[b] Canadian Food and Drug Directorate, Ottawa.
[c] Canada Department of Agriculture, Winnipeg.
[d] University of Minnesota.
[e] Fungi cultured for 1 week at 25 C and then 2 weeks at 12 C.

or its usefulness in screening production of several mycotoxins by representative fungi. Dense spore suspensions from fresh potato-dextrose-agar slopes were inoculated onto 5 ml of 2% yeast extract plus 15% sucrose medium [YES (2)] contained in a 30-ml vial (15). Cultures were incubated for 7 days at 25 C, except for the *Fusarium* species which usually required special conditions, and then extracted with hot chloroform. Each extract was concentrated to 0.5 ml before TLC. Where mycotoxin production on YES medium was successful, alternative media were in most cases not used. Otherwise, the following additional media were tried: Difco mycological broth plus 0.5% yeast extract (MYE), Czapek-Dox broth plus 0.5% yeast extract (CZ plus YE), and also moist rice in 250-ml flasks (for zearalenone production). Results are given in Table 2.

Since the fungi were known toxic producers or were of a type expected to produce the given mycotoxins, identification of the compounds was usually limited here to the two TLC solvent systems and one or more spray colors. Aflatoxins B_1 and G_1 from *A. sulphureus* were confirmed by formation of characteristic fluorescent derivatives on a microgram scale (7).

Although we were unable to obtain production of gliotoxin or zearalenone on YES medium, the value of this medium for screening toxinogenic fungi by the semimicro culture technique has been indicated.

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